Original Article

Clinical phenotypes and genetic analyses for diagnosis of systemic autoinflammatory diseases in adult patients with unexplained fever

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Abstract

Objective:

To make an accurate diagnosis of systemic autoinflammatory diseases (SAIDs), clinical and genetic analyses were performed in patients with unexplained fever.

Methods:

The clinical phenotype and genomic variants of 11 genes responsible for SAIDs were analyzed in 179 Japanese patients with unexplained fever. Genetic analysis was performed by next generation sequencing (NGS) on exons including exon-intron boundaries.

Results:

Three cases met the diagnostic criteria for SAIDs other than familial Mediterranean fever (FMF). Considering 176 patients with unexplained fever, 43 cases (24.0%) were clinically diagnosed as FMF. Gene variants were found in 53 cases (30.1%) when searching for variants in the 10 disease genes other than the *MEFV* gene. Among them, the most frequently-identified genes were *NLRP3*, *NOD2*, *NLRP12*, *NLRC4*, and *PLCG2*, which accounted for 14, 7, 17, 7, and 6 cases, respectively. These variants were rare, but not conclusive for diagnosis of its SAID.

Conclusion:

Twenty four percent of Japanese patients with unexplained fever were clinically diagnosed as FMF in this study. Low frequency but not disease-causing variants in genes other than *MEFV* were identified in 30.1% of the cases, and these gene variants might be associated with unexplained fever.

1. Introduction

Systemic autoinflammatory diseases (SAIDs) is a syndrome that inexplicably causes repeated systemic inflammation, often with fever, and inflammation of local sites such as joints, skin, intestines, and eyes [1]. Symptoms of SAIDs are similar to those of infections and collagen diseases, but no pathogenic microorganisms, autoantibodies, or antigen-specific T cells are detected [2, 3].

Genetic testing is required for accurate diagnosis of SAIDs. To date, the possible genes responsible for SAIDs have only been examined by the conventional Sanger sequencing, which makes diagnosis difficult when no gene variant is found and the possibility of other SAID cannot be ruled out. Therefore, we aimed to comprehensively analyze SAID genes by next generation sequencing (NGS) [4], and at the same time examine the clinical signs and symptoms of the selected SAID.

Familial Mediterranean fever (FMF), the most prevalent SAID in Japan, has recently been diagnosed [5] with a low frequency of exon 10 pathogenic variants and a high frequency of so-called non-exon 10 variants, such as exon 2 and exon 3 polymorphism [6]. Non-exon 10 variant is often a genetic polymorphism found in healthy individuals, resulting in debate on the diagnosis of FMF in patients possessing these variants [7-9]. Since FMF has been diagnosed with the clinical diagnostic criteria [10], it may be important to examine SAID genes other than *MEFV*, especially in cases diagnosed as FMF with non-exon 10 variants and without *MEFV* variants.

Several disease genes for SAIDs have been recently identified [11-13]. Fever is a common clinical symptom of SAIDs, and cases have been reported in which the clinical symptoms typical of the disease phenotype were not observed [14-16].

In this study, we examined the clinical symptoms of patients with unexplained fever, and at the same time, conducted comprehensive genetic analysis with NGS to evaluate the proportion of patients with FMF as well as the frequency of detecting *MEFV* variants. In addition, we identified variants in 10 SAID disease genes other than *MEFV*, and examined the proportion of non-exon 10 variants in patients diagnosed with FMF and the percentage of cases without *MEFV* variants.

2. Materials & methods

2.1 Patients

In total, 179 patients with unexplained fever treated between March 2011 and March 2017 at 56 Japanese hospitals, including Kurume University Hospital in Japan were enrolled in this study after taking the informed consent. We excluded 18 patients who were clinically diagnosed with other conditions, such collagen disease, infection, malignant tumor, immunodeficiency, myelodysplastic syndrome (MDS), and trisomy 8 (Figure 1). The 11 cases of collagen diseases included 4 cases of adult-onset Still's disease, 1 case of aortitis, 1 case of vasculitis, 1 case of spondyloarthropathy, 1 case of mixed connective tissue disease, 1 case of central nervous system lupus, 1 case of dermatomyositis, and 1 case of incomplete type of Bechet disease. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Kurume University (No. 337).

2.2 Genetic analysis

Blood from patients with unexplained fever was collected in EDTA-containing tubes, and DNA was extracted using the QIAmp DNA Blood Midi Kit (Qiagen, Waltham, USA). We investigated the following 11 genes related to SAIDs: *MEFV, TNFRSF1A, NLRP3, MVK, NOD2, IL1RN, NLRP12, PSTPIP1, PSMB8, NLRC4,* and *PLCG2.* Target sequencing of these genes was performed on an illumine MiSeq or NextSeq500 (Illumina, Inc., San Diego, USA) by amplicon sequencing [17,18] or hybridization-capture sequencing with predesigned capture probes for these genes (Integrated DNA Technologies, Inc., Coralville, USA) [19] at Kazusa DNA Research Institute (Kisarazu, Japan). We searched for allele frequencies of the detected missense variants in East Asia using the Exome Aggregation Consortium (ExAC) Browser (http://exac.broadinstitute.org/) and reported variants from the Infevers registry for FMF and hereditary inflammatory disorders variants (http://fmf.igh.cnrs.fr/ISSAID/infevers/). We classified the missense variants in less than 1% of healthy individuals as rare variants and those not described in the database (Infevers) as novel variants.

2.3 Statistical analyses

Data were analyzed using JMP version 11 (SAS Institute Inc., Cary, NC). Results were

expressed as the mean ± standard deviation for continuous variables. For quantitative data, the Wilcoxon rank sum test compared 2 independent groups. Comparisons for categorical variables were evaluated using the chi- square test (or Fisher's exact test when appropriate). All tests were two-sided, and a p value <0.05 was considered statistically significant.

3. Results

3.1 Clinical characteristics of patients with unexplained fever

Of the 179 patients with unexplained fever, 88.3% exhibited fever over 38℃. Moreover, 62.6% of patients had fever durations of less than 3 days. Other than fever, 37.4%, 21.8%, and 40.8% of patients reported abdominal pain, thoracic pain, and arthralgia, respectively. The usage rates of colchicine and corticosteroid were 39.7% and 22.3%, respectively (Table 1).

3.2 Comparison of clinical symptoms between FMF and Non-FMF patients

Three cases met diagnostic criteria for SAIDs, including 2 cases of tumor necrosis factor receptor-associated periodic syndrome (TRAPS) cases and one case of pyogenic arthritis-pyoderma gangrenosum-acne (PAPA) syndrome. Diagnosis of FMF among the 176 patients was made on 43 cases (24.0%) on the basis of Tel Hashomer criteria (Table 2) [10]. We compared the clinical features of 43 cases of FMF (FMF group) with those of 133 cases of non-FMF excluding the 3 cases of SAIDs other than FMF (non-FMF group). Compared with the non-FMF group, the FMF group had a significantly higher frequency of patients with fever duration of 3 days or less and a significantly lower frequency of patients with fever duration of 4 days or more ($p< 0.001$). In clinical symptoms other than fever, the FMF group tended to have significantly more abdominal and thoracic pain than the non-FMF group ($p< 0.001$). In terms of treatment history, the FMF group was significantly more likely to use colchicine than the non-FMF group ($p< 0.001$).

3.3 Comparison of MEFV variants in FMF and Non-FMF patients

Table 3 shows the results of variants in the *MEFV* gene among 11 disease genes associated with SAIDs. Exon 10 pathogenic variants were found in 9 cases, accounting for 20.9% of the 43 cases diagnosed as FMF according to Tel Hashomer criteria [10]. On the other hand, exon 10 pathogenic variants were not found in the non-FMF group. Among the 43 FMF cases, non-exon 10 variants were found in 20 cases (46.5%), and 14 FMF cases (32.6%) were without *MEFV* variants.

3.4 Analysis of 10 SAID genes other than MEFV in patients with unexplained fever

We analyzed 179 cases for variants in 10 SAID genes other than the *MEFV* gene. Among the 176 cases (excluding 2 cases of TRAPS and one case of PAPA syndrome that met the diagnostic criteria), we detected variants in *TNFRSF1A*, *NLRP3*, *NOD2*, *NLRP12*, *PSTPIP1*, *PSMB8*, *NLRC4*, and *PLCG2*, in 2, 14, 7, 17, 2, 2, 7, and 6 cases,

respectively. Almost all of gene substitution was missense variants as shown in Table 4. Splicing variant of *NLRP12* was found in one case. Finally, 53 cases (30.1%) exhibited variants in 10 SAID genes other than the *MEFV* gene when duplicate cases were considered. No variants were found in *MVK* and *IL1RN* genes. Cases with gene variants in *NLRP3* and *NLRP12* exhibited abdominal pain and arthralgia, and cases with gene variants in *NOD2* tended to have myalgia (Table 4). Furthermore, we examined whether these cases were actually diagnosed as FMF on the basis of Tel Hashomer criteria [10]. Patients with *NLRP3*, *NOD2*, and *NLRP12* variants were diagnosed with FMF in 3 out of 14 cases (21.4%), 2 out of 7 cases (28.6%), and 3 out of 17 cases (17.6%), respectively. Patients with *NLRC4* and *PLCG2* variants were not diagnosed with FMF. Nine out of 57 cases (15.8%) with variants in 10 genes other than *MEFV* were clinically diagnosed as FMF.

3.5 Variants in 10 SAID genes in FMF diagnosed cases excluding Exon 10 variants

As shown in Table 3, 43 cases were diagnosed as FMF; 9 complete FMF cases (20.9%) with exon 10 pathogenic variants, and the other 34 cases had variants in exon 2 and/or exon 3 (non-exon 10 variants) or no variants in *MEFV*. There were 20 cases with non-exon 10 variants (46.5%) and 14 cases without *MEFV* variants (32.6%). Since we might miss disease-causing variants of other SAID gene, we further investigated other 10 SAID genes in 34 cases diagnosed as FMF. Rare variants were detected in 6 of 34 cases (17.6%); 4 out of 20 cases with non-exon 10 variants (20.0%) and 2 out of 14 cases without *MEFV* variants (14.3%). Among the 6 cases with these gene variants, the *NLRP3* variant was the most common (3 cases), followed by *NLRP12* variant (2 cases), and *NOD2* and *PSTPIP1* variants (1 case each) (Table 5).

4. Discussion

Fever of unknown origin is unavoidable in daily medical care; however, SAIDs such as FMF have been increasingly reported in Japan [5], resulting in the need to identify genes responsible for SAIDs to make diagnosis of patients with unknown and unexplained fevers. Previously, the Sanger method was used to perform genetic tests for a presumed SAID gene, but searching for other possible disease genes was time-consuming if no variant was found in the targeted gene. As shown in this study, a comprehensive analysis of SAID genes in patients with fever of unknown origin using NGS has the benefit of reducing diagnosis lag as the number of SAIDs increases. Furthermore, more accurate data such as mosaicism and copy number variant analysis can be extracted compared with the Sanger method, which is advantageous for cost reduction [18, 19].

FMF is the most frequently diagnosed SAID in Japan. Of the 43 cases in our study diagnosed as FMF, 83.7% had fever duration of less than 3 days, which is characteristic of FMF. Moreover, the frequency of abdominal and thoracic pain in the FMF group was significantly higher compared with the non-FMF group. The efficacy of colchicine is used as a therapeutic diagnosis when FMF is suspected. Therefore, the frequency of colchicine treatment in the FMF group may have been significantly higher than expected. In the analysis for *MEFV* as the responsible gene for FMF, all the cases with exon 10 pathogenic variants were diagnosed as FMF. Moreover, 32.6% of FMF patients met the diagnostic criteria for FMF without *MEFV* variants identified by NGS. This frequency was higher than reported in Japanese epidemiological survey [20]; however, some previous reports have exceeded 30% [21, 22], which suggested that it was likely due to differences in the populations studied.

Interestingly, there was one S242R heterozygous case in our *MEFV* analysis [23]. This pathogenic variant can cause a recently reported disease called pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) [24]. Although the variant residing in the exon 2 of *MEFV*, the clinical phenotype differed from FMF and thus this case was not diagnosed as FMF. The term pyrin-associated autoinflammatory disease (PAAD) has been proposed to describe *MEFV*-related autoinflammatory syndromes, including FMF and PAAND [25].

In the search for disease-causing variants in 10 disease genes other than *MEFV*, diagnosis was not made for cryopyrin-associated periodic syndromes (CAPS) [26], Blau syndrome [27], familial cold-induced autoinflammatory syndrome 2 (FCAS2) [28], *NLRC4*-associated macrophage activation syndrome [29, 30], and PLAID/APLAID [31, 32] with variants in responsible genes *NLRP3*, *NOD2*, *NLRP12*, *NLRC4*, and *PLCG2,*

respectively. Furthermore, patients with *NLRP3*, *NOD2*, and *NLRP12* variants were diagnosed with FMF at low frequencies of 21.4%, 28.6%, and 16.7%, respectively, and patients with *NLRC4* and *PLCG2* variants were not diagnosed with FMF (Table 4). In recent years, there have been reported cases of SAIDs that exhibit various symptoms associated with the identified disease gene other than the main clinical symptoms of SAIDs [14-16]. There are reports that *NLRP3* variants with low-penetrance account for 16% of cases called "undefined autoinflammatory disease" that cannot be diagnosed as CAPS [15]. Especially, the *NLRP3* V198M variant shows atypical phenotypes compared to CAPS and low-penetrance [33]. *NOD2*-associated autoinflammatory disease (Yao syndrome) has *NOD2* variants and various symptoms such as fever, dermatitis, polyarthralgia and joint swelling mainly in the lower limbs, digestive symptoms, and dry symptoms [34]. *NLRP12* autoinflammatory disease (NLRP12-AD) has *NLRP12* variants and exhibits various symptoms such as fever, chest pain, abdominal pain, and headache, in addition to cold urticaria [14, 35]. *NLRC4*-related autoinflammatory disease has *NLRC4* variants and clinical symptoms of fever similar to CAPS, urticaria-like eruption, inflammatory bowel disease and macrophage activation syndrome [29, 30]. In some cases, severe inflammatory bowel disease and macrophage activation syndrome are not complicated [36]. In the cases in this study, clinical symptoms of the typical *NLRP3* variant, Yao syndrome, NLRP12-AD, *NLRC4*-related autoinflammatory disease were not observed. However, because many of these diseases have symptoms of fever, these SAID gene variants may be associated with fever. Ter Haar and colleagues [37] conducted a clinical and genetic analysis on 187 cases with fever but no diagnosis of SAIDs as "undefined autoinflammatory disease". The most common symptoms in addition to fever were arthralgia, myalgia, and abdominal pain, which was similar to our study. The researchers stated that the lack of complete genetic analysis was a challenge [37]. With regard to treatment, it is difficult to judge whether patients with unexplained fever as the main symptom should be treated the same as each SAIDs patient whose diagnosis is confirmed. Further case accumulation and prospective follow-up including treatment response may be necessary for this decision.

FMF, which is the most common SAIDs, is less frequently diagnosed in Japan than in the United States and Europe, with a high frequency of so-called non-exon 10 *MEFV* variants, such as exon 2 and exon 3 polymorphisms [6]. Non-exon 10 variant is often a genetic polymorphism found in healthy individuals, and there is much debate on its inclusion in FMF diagnostic criteria [7-9]. In recent years, a couple of attempts have been made to review the diagnostic criteria of SAIDs [38, 39]. In particular, FMF has complicated diagnostic criteria and hence, simplification is warranted. Among 34

clinically diagnosed cases of FMF with non-exon 10 variants or without *MEFV* variants in our study, SAID genes variants other than *MEFV* were detected in 6 cases (17.6%)(Table 5). In these 6 cases, *NLRP3*, *NLRP12*, *NOD2*, and *PSTPIP1* variants were observed, and these gene variants may affect the FMF phenotype expressed.

In this study, 10 autoinflammatory disease genes other than *MEFV* were searched, and rare variants which were not disease-causing were detected in 53 cases (30.1%). However, even if 10 genes were searched, the detection rate was lower than expected. In addition, only 3 cases of 10 autoinflammatory diseases other than FMF were diagnosed. One reason may be that the subjects who entered the study had few children and many adults. In order to accurately diagnose unexplained fever, it is necessary to further expand the number of genes associated with SAIDs to be analyzed in the future and employ NGS for comprehensive analysis [4]. Recently, digenic inheritance (DI) in autoinflammation-associated genes is attracting attention [40] and software that predicts DI is also being developed [41-43]. Considering DI, it is meaningful to comprehensively search for as many autoinflammatory disease genes as possible. Currently, a panel of autoinflammatory diseases is being created and 25 autoinflammatory disease genes are planned, but genes that are expected to be less frequent are added, so increasing the number of genes could huge impact on the detection rate is suspicious. Since increasing the number of genes also costs, it is necessary to further examine what kind of appropriate diagnostic scheme should be applied in the future.

In summary, 24.0% of Japanese patients with unexplained fever were clinically diagnosed with FMF in this study. Variants in 10 SAID genes other than *MEFV* were found in 30.1% of cases, and these variants may be associated with unexplained fever. There are many phenotypes of SAIDs that cause fever, suggesting the significance of comprehensive gene analysis by NGS for improved diagnosis lag, accurate data, and cost reduction compared with the Sanger method.

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Conflict of interest

None

Figure legends

Figure 1. Flow diagram of patient enrollment.

FMF; Familial Mediterranean fever. TRAPS; TNF receptor-associated periodic syndrome. PAPA; pyogenic arthritis, pyoderma gangrenosum, acne. MDS; Myelodysplastic syndrome

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Figure 1

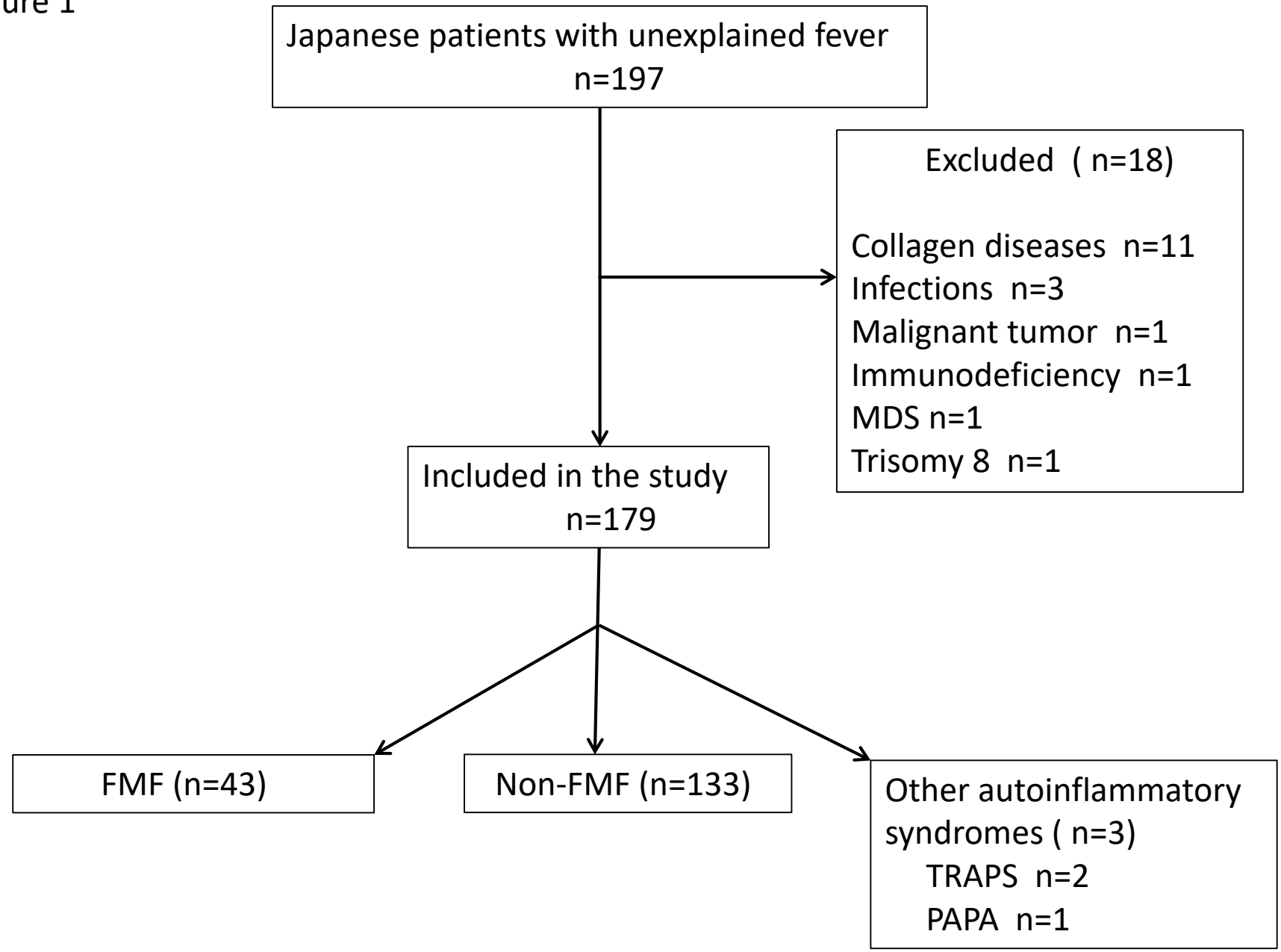
Japanese patients with unexplained fever
n=197
Excluded (n=18)
Collagen diseases n=11
Infections n=3
Malignant tumor n=1
Immunodeficiency n=1
MDS n=1
Trisomy 8 n=1
Included in the study
n=179
FMF (n=43)
Non-FMF (n=133)
Other autoinflammatory
syndromes (n=3)
TRAPS n=2
PAPA n=1

Table 1 Clinical features of patients with unexplained fever (n=179)

Age	39.2±17.4 (0.4-81)
Age at onset	32.9±19.5 (0-80)
Male/female	82/97
Symptoms	
Fever	179 (100)
Degree	
Low (<38℃)	21 (11.7)
High (≥38℃)	158 (88.3)
Duration	
≤ 3 days	112 (62.6)
≥ 4 days	67 (37.4)
Frequencies	
> 1/month	99 (55.3)
< 1/month	80 (44.7)
Abdominal pain	67 (37.4)
Thoracic pain	39 (21.8)
Arthralgia	73 (40.8)
Myalgia	32 (17.9)
Erysipelas-like erythema	15 (8.4)
Treatment	
Colchicine	71 (39.7)
Corticosteroid	40 (22.3)

Table 2. Comparisons of clinical features between FMF and non-FMF

	FMF	non-FMF	*p-value (FMF vs non-FMF)*
Number	43	133	
Age	37.4±16.1	39.9±17.7	0.472
Age at onset	31.5±18.6	33.4±20.0	0.623
Male/female	14/29	66/67	0.051
Symptoms			
Fever	43(100)	133(100)	NS*
Degree			
Low (<38℃)	4(9.3)	17(12.8)	0.599
High (≥38℃)	39(90.7)	116(87.2)	
Duration			
≤3 days	36(83.7)	73(54.9)	<0.001
≥4 days	7(16.3)	60(45.1)	
Frequencies			
> 1/month	30(69.8)	76(57.1)	0.412
< 1/month	13(30.2)	57(42.9)	
Abdominal pain	26(60.5)	40(30.1)	<0.001
Thoracic pain	21(48.8)	17(12.8)	<0.001
Arthralgia	14(32.6)	57(42.9)	0.284
Myalgia	5(11.6)	25(18.8)	0.354
Erysipelas-like erythema	4(9.3)	10(7.5)	0.748
Treatment			
Colchicine	27(62.8)	44(33.1)	<0.001
Corticosteroid	8(18.6)	29(21.8)	0.823

*NS, not significant

Table 3. *MEFV* genotypes in FMF or non-FMF patients

		FMF	non-FMF
Exon	**Mutation**		
Exon 10	M694I/E148Q	8(18.6)	0
	M694I/normal	1(2.3)	0
	sub total	9(20.9)	0
Non-Exon 10	S503C/ R408Q/P369S/ E148Q	0	1(0.8)
	S503C/R202Q	0	1(0.8)
	S503C/E148Q	0	2(1.5)
	S503C/normal	1(2.3)	5(3.8)
	R410H/E84K	0	1(0.8)
	R408Q/P369S	1(2.3)	5(3.8)
	R408Q/P369S/ E148Q/E148Q/L110P	0	1(0.8)
	R408Q/P369S/ E148Q	4(9.3)	9(6.8)
	P369S/E148Q	0	2(1.5)
	P369S/E148Q/L110P	0	1(0.8)
	G304R/R202Q	0	1(0.8)
	G304R/normal	0	5(3.8)
	S242R/E148Q	0	1(0.8)
	R202Q/normal	4(9.3)	3(2.3)
	E148Q/E148Q	2(4.7)	1(0.8)
	E148Q/E148Q/L110P	1(2.3)	2(1.5)
	E148Q/L110P	5(11.6)	6(4.5)
	E148Q/normal	2(4.7)	30(22.6)
	E84K/normal	0	1(0.8)
	sub total	20(46.5)	78(58.6)
No mutation	-	14(32.6)	55(41.4)
Total		43(100)	133(100)

Table 4. Analysis of 10 SAID genes other than *MEFV* in patients with unexplained fever

Gene name	*TNFRSF1A*	*NLRP3*	*NOD2*	*NLRP12*	*PSTPIP1*	*PSMB8*	*NLRC4*	*PLCG2*	*MVK*	*IL1RN*	Total
Number	2	14	7	17	2	2	7	6	0	0	57
Gene Substitution											
Variant											
missense	2	14	5	13	2	2	7	4			49
frame shift (FS)			1	1							2
missense+FS				2							2
nonsense			1								1
deletion								2			2
Splicing				1							1
Symptoms											
Fever	2(100)	14(100)	7(100)	17(100)	2(100)	2(100)	7(100)	6(100)	-	-	57(100)
Abdominal pain	0(0)	7(50)	2(28.6)	9(52.9)	0(0)	2(100)	2(28.6)	2(33.3)	-	-	24(42.1)
Thoracic pain	1(50)	5(35.7)	2(28.6)	3(17.6)	0(0)	1(50)	1(14.3)	2(33.3)	-	-	15(26.3)
Arthralgia	1(50)	6(42.9)	2(28.6)	6(35.3)	1(50)	2(100)	2(28.6)	2(33.3)	-	-	22(38.6)
Myalgia	1(50)	2(14.3)	3(42.9)	2(11.8)	1(50)	1(50)	1(14.3)	3(50)	-	-	14(24.6)
Erysipelas-like erythema	0(0)	3(21.4)	2(28.6)	1(5.9)	0(0)	0(0)	0(0)	0(0)	-	-	6(10.5)
Diagnosis											
FMF	0(0)	3(21.4)	2(28.6)	3(17.6)	0(0)	1(50)	0(0)	0(0)	-	-	9(15.8)
non-FMF	2(100)	11(78.6)	5(71.4)	14(82.4)	2(100)	1(50)	7(100)	6(100)	-	-	48(84.2)

Table 5　10 SAID gene variants in FMF patients with non-exon 10 variants and without *MEFV* variant

No.	*MEFV*	*TNFRSF1A*	*NLRP3*	*NOD2*	*NLRP12*	*PSTPIP1*	*PSMB8*	*NLRC4*	*PLCG2*	*MVK*	*IL1RN*
Non-Exon 10 (n=20)											
1.	R202Q/normal	-	R731W	-	-	-	-	-	-	-	-
2.	E148Q/E148Q	-	G811S	-	-	-	-	-	-	-	-
3.	E148Q/L110P	-	-	-	P210L	-	-	-	-	-	-
4.	E148Q/L110P	-	-	-	-	D289N	-	-	-	-	-
No mutation (n=14)											
5.	-	-	V72M, M637T	-	-	-	-	-	-	-	-
6.	-	-	-	A110T	F402L	-	-	-	-	-	-